Bilateral brain activity in auditory regions is necessary for successful vocal learning in songbirds

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SUPPLEMENTARY METHODS

Song analysis

Songs from the afternoon on day 120 were sorted based on the length of the sound file as to exclude empty files or files that contained just one call or song motif. We only used sound files that contained at least two song motifs, and sound files with noise artifacts such as cage noises were excluded from further analysis. Of the sound files that met these criteria, we randomly chose 20 files for analysis. We consistently analysed the second motif in a song file, which is not preceded by introductory notes. The songs from the tutors and experimental birds were band-pass filtered (>400 Hz) and equalized for RMS amplitude using Praat software. Sound Analysis Pro [1] was used to assess the fidelity of tutor song imitation of the experimental subjects by calculating the "percentage similarity" to their tutor with the default settings for zebra finches. Similarity scores generated by this software are based on three major components: Euclidean distances between acoustic features (wiener entropy, spectral continuity, pitch, and frequency modulation), percent similarity score computed over long intervals (50-70ms), and accuracy measured over short intervals of time (5-10ms). Sound Analysis Pro has a "floor effect," because zebra finch songs always resemble each other somewhat on these features. We thus compared songs from birds in the main experiment with songs from unfamiliar birds (that is, birds that were not genetically related to any of the subjects or their tutors, and that were not present in our colony when these birds or their tutors were born) to investigate whether the juveniles had learned from their song tutors specifically or if similarity was attributable to general song characteristics. To introduce as much variability as possible into the novel song comparisons, each experimental bird's songs were compared to five novel birds' songs (four songs per novel bird for twenty song comparisons total). Songs from unfamiliar birds were selected to represent song diversity (song files with recording artifacts such as cage noises were not used), as to get an average measure of 'novel song similarity'. The same five novel birds were used for all experimental subject comparisons. These 20 comparisons for TUT and for NOV generated one average TUT-song-similarity and one average NOV-song-similarity score for each bird.

SUPPLEMENTARY RESULTS

Histology

We confirmed that cannulas used for injecting TTX were targeted to the auditory lobule in parasagittal sections incubated against *egr-1* (Suppl. Fig. 2B, C). At 150 dph visual inspection of

sections confirmed that there was *egr-1* expression throughout the NCM, surrounding the cannula tracks. Thus, the neural tissue that was subjected to TTX earlier in development showed a molecular neuronal response to song playbacks three months after infusions had been completed (Suppl. Fig. 2B).

In order to investigate whether there was a correlation between strength of song learning and neuronal activation in the experimental or control hemisphere [2-5], we counted *egr-1* positive nuclei in images acquired with a 20x objective. However, because the cannulas created tracks through the NCM that were permanent in the image, a quantitative analysis of *egr-1* expression immediately surrounding the tracks was unreliable. Indeed, the area in each image that remained for analysis with the magnification needed to count cell nuclei was not sufficiently large and varied too much between subjects. We thus only performed visual inspection of whether there was *egr-1* expression surrounding the tracks.

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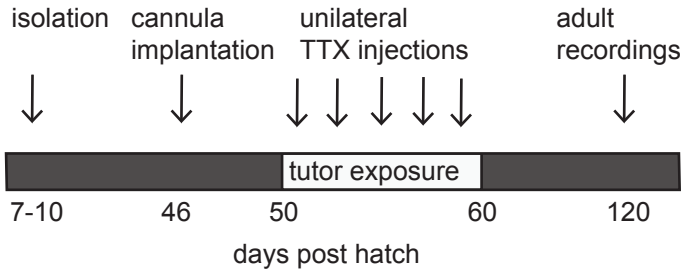
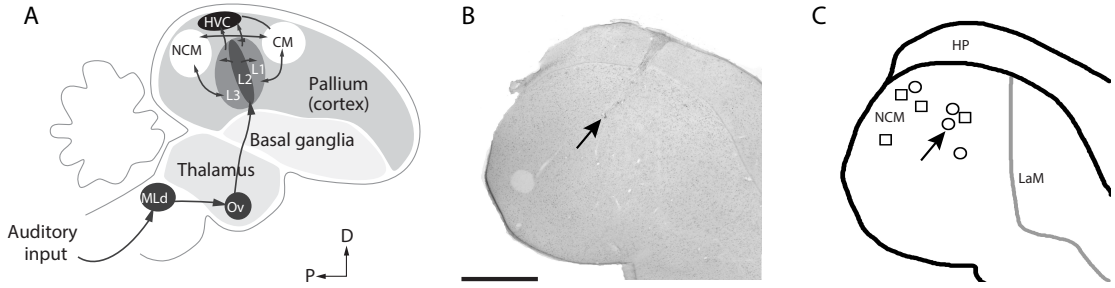
SUPPLEMENTARY FIGURES AND TABLES

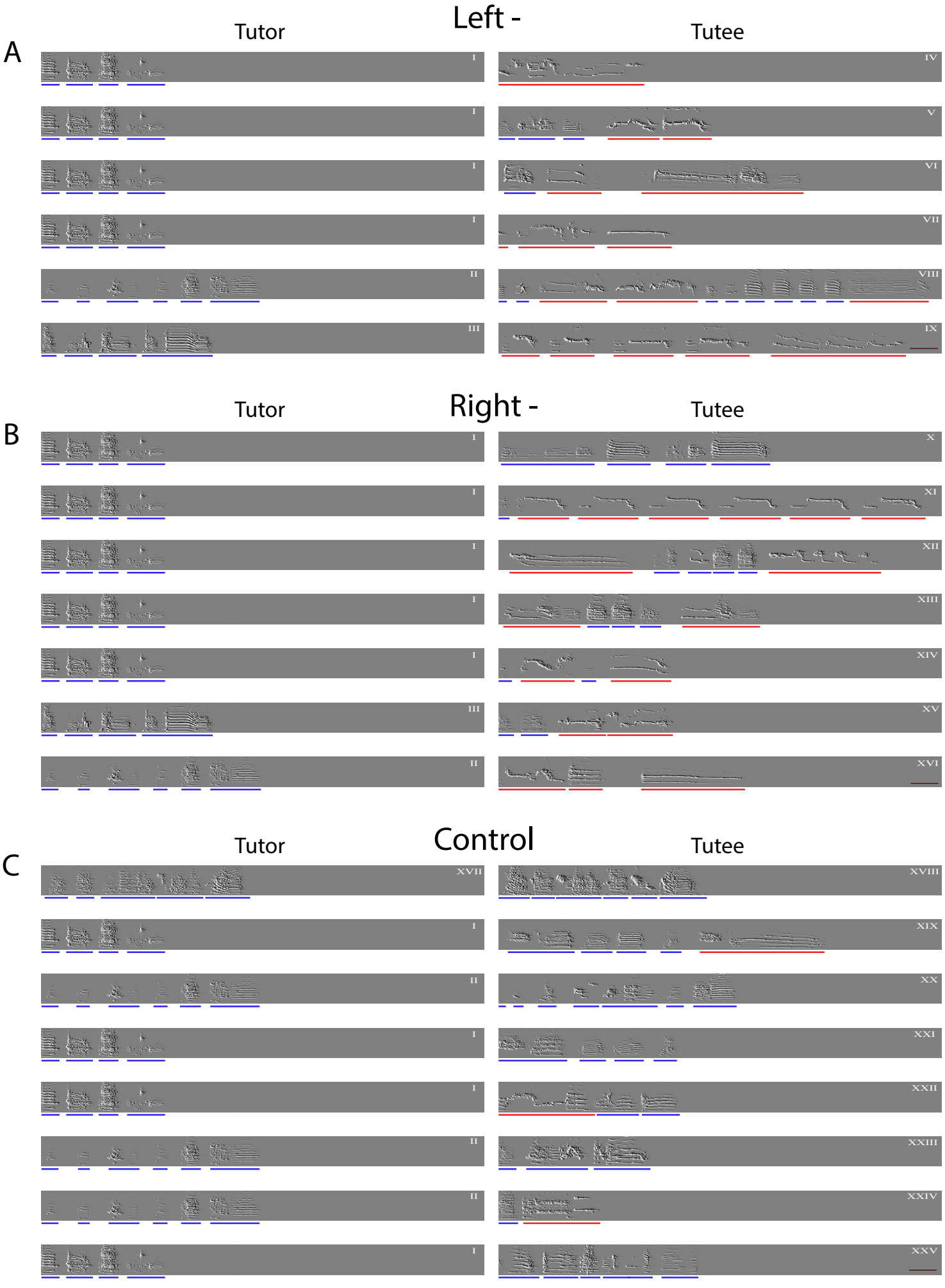


Supplement figure S1: Experimental timeline. Juvenile male zebra finches were raised in isolation to control social and auditory exposure to song. At 46 dph, birds were surgically implanted with cannulas to infuse TTX unilaterally into the NCM. During the peak of the sensitive period for song learning (between 50-60 dph), birds received 5 TTX injections targeted to either the left or right hemisphere NCM. Each injection was immediately followed by a 90-minute tutoring session (see Methods). Songs were recorded at 120 dph to assess quality of song imitation.



Supplement figure S2: Diagram of the songbird auditory pathway and summary of histological results. (A) Simplified diagram (not to scale, medial and lateral planes collapsed to show all relevant structures and their connections) of the avian auditory system. The ascending auditory pathway (dark gray arrows) includes a midbrain nucleus (MLd), a thalamic nucleus (Ov) and several cortical regions. The primary auditory cortex (Field L2) connects to the secondary auditory regions Field L3 and L1, which in turn are reciprocally connected to regions analogous to the mammalian auditory association cortex (NCM and medial and lateral CM). Connections between the auditory pathway and HVC in the song system are also shown. Adapted, with permission from: Chirathivat et al., 2015. (B) Photomicrograph of the auditory lobule in a parasagittal section incubated against egr-1 illustrating the track left by the cannula (projection depth of syringe indicated by black arrow). Note that in this medial section, Field L, which can be identified by a complete lack of *egr-1* expression, is not present (the perfectly circular spot on the

ventral side of NCM is a processing artifact). (C) Graphic representation of the auditory lobule in parasagittal view indicating locations of injection sites. Black arrow indicates the location of injection from the animal shown in (B). Circles and squares represent left and right TTX injected birds, respectively. Tracks could not be located for 5 birds (2 left-TTX-injected, 3 right-TTX-injected) due to technical problems during sectioning. Abbreviations: HP, hippocampus; LaM, lamina mesopallialis. Scale bar represents 1 mm.


Left -
Tutor
Tutee
A
I
IV
I
V
I
VI
I
VII
II
VIII
III
IX
Right -
Tutor
Tutee
B
I
X
I
XI
I
XII
I
XIII
I
XIV
III
XV
II
XVI
Control
Tutor
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C
XVII
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XXII
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XXIII
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XXV

Supplement figure S3: Representative examples of song spectrograms of all song tutors (left panels) and experimental birds (right panels; (A) left-TTX infused, (B) right- TTX infused, and (C) control group). Roman numerals on the spectrograms indicate specific birds. One motif from each tutee (right) and their respective tutor (left) is represented. Normal-appearing syllables are indicated with blue bars, and isolate-like syllables with red bars. Song recordings used for creating each spectrogram are linked to this figure and will play automatically when the figure is opened in Adobe Acrobat and the cursor is moved over the spectrogram. Scale bar represents 100ms.

Supplement Table 1: Similarity scores to tutor and novel and mean values of five acoustic features (Pitch (kHz), frequency modulation (FM), amplitude modulation (AM), entropy, and goodness of pitch) for all experimental animals.

Group	Tutor sim(%)	Novel sim(%)	Imitation score (TUTsim-NOVsim)	Pitch (kHz)	FM	AM (Hz)	Entropy	Goodness of Pitch
TTX-Left	10.12	2.56	7.56	2.9	36.83	-1.01E-03	-3.80E+00	179.59
TTX-Left	19.3	18.88	0.42	1.9	34.93	-7.93E-04	-3.41E+00	246.88
TTX-Left	40.7	43.44	-2.74	1.2	28.74	-1.08E-03	-3.67E+00	463.96
TTX-Left	7.88	1.68	6.2	3.9	35.85	-2.77E-03	-4.49E+00	122.74
TTX-Left	60	47.84	12.16	2.0	36.89	-7.82E-04	-3.20E+00	312.76
TTX-Left	42.61	19.68	22.93	3.3	33.89	-6.94E-04	-4.29E+00	177.82
TTX-Right	65.28	41.32	23.96	0.6	26.17	-8.60E-04	-2.98E+00	451.69
TTX-Right	21.72	16.44	5.28	1.8	33.60	-7.79E-04	-3.55E+00	254.90
TTX-Right	40.45	19.96	20.49	3.8	32.73	-5.67E-04	-4.37E+00	165.24
TTX-Right	52.06	44.64	7.42	1.2	28.9	-5.05E-04	-3.31E+00	378.51
TTX-Right	25	15.88	9.12	1.8	30.95	-1.98E-03	-3.26E+00	241.23
TTX-Right	30.44	16.24	14.2	1.9	34.87	4.93E-04	-3.07E+00	391.64
TTX-Right	26.6	7.28	19.32	2.0	26.23	-3.35E-04	-4.24E+00	169.94
Control	63.53	35.84	27.69	1.0	25.93	-4.40E-04	-2.98E+00	299.45
Control	80.72	40.84	39.88	1.0	34.67	-1.36E-03	-2.77E+00	262.21
Control	72.08	24.28	47.8	1.8	31.51	-1.12E-03	-2.87E+00	137.67
Control	57.2	15.8	41.4	1.8	30.15	-4.71E-04	-3.50E+00	208.76
Control	51.12	14.64	36.48	0.9	36.40	-6.26E-04	-2.89E+00	234.53
Control	37.68	6.2	31.48	2.1	32.05	-8.17E-04	-3.12E+00	132.30
Control	72.52	45.24	27.28	0.7	30.01	-1.36E-03	-2.43E+00	475.54
Control	82.68	37.29	45.39	0.9	39.58	-9.33E-04	-2.34E+00	370.33

Supplement Table 2: Composition of clutches used in the study.

Subject	Clutch size	Breeding Pair
1	3F, 1M	Pair 1
2	2F, 1M	Pair 2a
3		
4	3F, 1M	Pair 3
5	2F, 3M	Pair 4
6		
7		
8	1F, 3M	Pair 2b
9		
10		
11	3M	Pair 5
12		
13		
14	3M	Pair 6
15		
16		
17	2F, 2M	Pair 7
18		
19	1F, 2M	Pair 8
20		
21	2F, 2M	Pair 9
-	2F, 1M	Pair 10
-	1M	Pair 11
-	1F, 2M	Pair 12